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Award Number: W81XWH-07-1-0582

TITLE: Differential Phosphoprotein Profiling of Tamoxifen Response

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REPORT DATE: August 2009

TYPE OF REPORT:Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-08-2009	Annual	7/30/2008-7/29/2009

4. TITLE AND SUBTITLE
Differential Phosphoprotein Profiling of Tamoxifen Response

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0582

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Chicago

Chicago, IL 60637

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Advanced breast cancers that initially respond well to tamoxifen treatment eventually become refractory to this compound. Several mechanisms of acquired resistance have been hypothesized, including crosstalk between ER and growth factor receptor tyrosine kinase pathway. The cumulative data from clinical studies show that overexpression of HER-2 and/or EGFR, and high levels of phosphorylated Akt or ERK, contribute to tamoxifen resistance in some patients. HER-2, EGFR, Akt and ERK are all kinases and components of signaling pathways critical to cell growth and survival, highlighting the need for global phosphoproteome analysis. I have developed a method for comparison of global phosphoprotein profiles involving stable isotope labeling, a phosphoprotein affinity step, 1-D SDS-PAGE and LC-MS/MS. I identified 26 proteins that respond to tamoxifen differently in MCF-7 (tamoxifen sensitive) and MCF-7/HER2-18 (tamoxifen resistant) cells. FADD and PAK1 have previously been described as being involved in generation of tamoxifen resistance showing that phosphoprotein profiling is capable of identifying proteins relevant to tamoxifen resistance. We also observed a striking synergistic interaction between ionizing radiation (IR) and tamoxifen in a model for combining high dose radiation therapy (RT) with hormone therapy for breast cancer and propose the use of tamoxifen as a radiosensitizer during radiation treatment.

15. SUBJECT TERMS
tamoxifen resistance, proteomics, biomarker, phosphorylation, estrogen receptor, breast cancer

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	21	USAMRMC

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Introduction

Breast cancer remains the most common malignancy affecting women in the United States. About 80% of breast cancers are estrogen-receptor-alpha-positive (ERα+), some of which respond to estrogen hormone therapy. ERα is a ligand-activated transcription factor that plays a critical role in the etiology of breast cancer [1-3]. Selective estrogen receptor modulators (SERMs) have variable agonistic and/or antagonistic activities, depending on the type of ER (α versus β), tissue context, and interactions with different proteins such as transcriptional co-activator or co-repressors [4]. The first SERM, tamoxifen, revolutionized breast cancer treatment when it came into use some three decades ago. In ERα breast cancer cells, tamoxifen blocks cancer growth by competing for binding to ER and cuts recurrence risk in half [5] [6]. More recently, tamoxifen has been shown to prevent breast cancer in high-risk women [7] [8]. Even in patients with ERα-positive breast cancer, only 40–50% of patients benefit from tamoxifen treatment, suggesting that a substantial fraction of ER-positive cancers are resistant to this drug. Additionally, advanced breast cancers that initially respond well to tamoxifen eventually become refractory to this compound. In some cases, tamoxifen can even act as a growth stimulatory signal. Several mechanisms of resistance have been hypothesized, including crosstalk between ER and other proliferative signals, such as growth factor receptor tyrosine kinase pathways [9-12]. The cumulative data from clinical studies show that overexpression of HER-2 and/or EGFR, and high levels of phosphorylated Akt or ERK, contribute to tamoxifen resistance in some patients [13-16]. HER-2, EGFR, Akt and ERK are all kinases and components of signaling pathways critical to cell growth and survival, highlighting the need for global phosphoproteome analysis.

Although many biomarkers for breast cancer prognosis and therapy initially appeared attractive, over the years most of them have failed to become clinically useful, with the exception of hormone receptors (ER and PR) and the HER-2 tyrosine kinase receptor [17, 18]. Although ER status provides prognostic information, the major clinical value is to assess the likelihood that a patient will respond to endocrine therapy [2, 19]. HER2 is overexpressed in 25 to 30 percent of breast cancers, increasing the aggressiveness of the tumor [20]. The drug Trastuzumab (Herceptin) is a monoclonal antibody directed against the HER-2 and has a survival benefit when combined with chemotherapy in patients with metastatic breast cancer that overexpress HER-2 [21]. However, tumors that overexpress HER2 tend to be ERα negative and thus represent a separate treatment group. Current prognostic classifications are thus not enough to represent the broad clinical heterogeneity of breast cancer, making it difficult to target therapeutic strategies to each patient. A major component of prognosis for patients undergoing endocrine therapy is the acquired resistance to tamoxifen. Finding biomarkers for tamoxifen resistance and/or drugs that could help overcome the resistance is a very important topic.

New reporters that could be used in combination with existing markers for screening of breast cancer cells for treatment decisions or to predict therapy outcome are still needed. A major component of prognosis for patients undergoing endocrine therapy is the acquired resistance to tamoxifen. Finding reporters for tamoxifen

resistance and/or drugs that could help overcome the resistance is a very important topic.

Thanks to recent advances in technology and the ability to analyze enormous amounts of data, proteomics is poised to have a significant effect on cancer research. Although gene expression patterns of cancerous cells have been extensively studied, there is a dearth of information on protein expression and protein modification patterns. This is important because gene expression alone cannot determine the activation state of cellular proliferation signaling pathways. Aberrations in the regulation of these pathways are a key to the development and progression of cancers. The activity of signaling proteins depends on their interactions with other proteins and modifications (phosphorylations) they undergo over time, areas that proteomics is able to address [22, 23].

Before starting this project, I had developed and published a method for enrichment of phosphoproteins [24]. The methodology involves a phosphoprotein affinity step, 1-dimensional SDS-PAGE and ESI LC-MS/MS and is termed PA-GeLC-MS/MS. By combining the phosphoprotein enrichment method with stable isotope labeling relative quantitation of phosphoprotein profiles can be obtained. The overall goal of this project is obtain global phosphoprotein profiles of tamoxifen response and to compare responses in tamoxifen sensitive and resistant cell lines. In this final report I describe phosphoprotein profiling of MCF-7 (tamoxifen sensitive) and MCF-7/HER2-18 (tamoxifen resistant) cells and report several proteins that respond differently to tamoxifen treatment in these two cell lines.

DNA damage, checkpoints, senescence and protein phosphorylation The potentially lethal form of damage-induced by ionizing radiation is DNA double strand breaks (DSBs). Throughout the cell cycle, DSBs can be repaired by non-homologous end-joining (NHEJ) while in late S or G2 phases, some may be repaired by homologous recombination (HR). However, RT induces tens of DSBs at a time, many of which may be "unrepairable". Persistent DNA damage over a number of days, whether due to eroded telomeres or unrepaired DSBs, can induce apoptosis, mitotic catastrophe and/or cell senescence. Current understanding links DNA damage-induced senescence to persistent checkpoint signaling and activation of the p53/INK/ARF/CIP pathway [25]. Tumor cell senescence, like apoptosis, has been proposed as a desirable outcome of RT and chemotherapy [26, 27] but agents that promote senescence have yet to be described. Estrogen was recently shown to decrease IR induced senescence [28]. Thus, we decided to test if tamoxifen could inhibit IR induced senescence and potentially be used as a radiosensitizer.

Body

PHOSPHOPROTEIN ENRICHMENT FROM CONTROL AND TAMOXIFEN TREATED MCF-7 AND MCF-7/HER2-18 CELLS

Phosphoprotein profiling was performed on two cell lines. First, the MCF-7 breast cancer cell line is estrogen receptor positive, responds to estrogen stimulation and is sensitive to tamoxifen. Several cell lines have been generated that are resistant to tamoxifen treatment. As mentioned previously, overexpression of HER2 has been described in patients with acquired tamoxifen resistance [29]. The tamoxifen resistant cell line used in these experiments, MCF-7/HER2-18, was generated by overexpressing full-length HER2 kinase in MCF-7 cells. The authors tested for response to tamoxifen by implanting MCF-7/HER2-18 or MCF-7 control cells into nude mice. Both cells only produced tumors when stimulated with estrogen, but MCF-7/HER2-18 grew much more rapidly. Tamoxifen inhibited growth in the MCF-7-derived tumors but not in the MCF-7/HER2-18 derived tumors [20].

Phosphoprotein enrichment experiments were performed on both MCF-7 (tamoxifen sensitive) cells and MCF-7/HER2-18 (tamoxifen resistant) cells (Figure 1, see next page). The cells were SILAC labeled with DMEM-Flex media (Invitrogen) without phenol red and contained high glucose (4500 mg/ml), 1mM sodium pyruvate, 10% heat-inactivated dialyzed fetal bovine serum, 1% penicillin/streptomycin and 0.3 mg/ml L-glutamine. Briefly, two equal amounts of cells were seeded onto plates, one was grown in “light” (L-lysine and L-Arginine) and the other in “heavy” ($^{13}C_6$ L-lysine and $^{13}C_6{}^{15}N_4$ L-Arginine) media for >10 doublings.

Prior to treatment cells were serum starved for 2 hours. The cells were then treated for 30 minutes with 10 nM 4-hydroxy-tamoxifen (Sigma) or ethanol as control. Whole cell lysates were prepared from 7 x 10^7 cells in 1.5 ml of lysis buffer (ProQ lysis buffer with 1 µM sodium fluoride, 1 µM okadaic acid and 0.1 µM sodium orthovanadate). The supernatant was collected, and protein yields were determined by Bradford analysis using Bio-Rad protein assay reagent. About 5 mg of lysate was obtained from each sample. A sample of the lysate was stored for follow-up analysis using Western blots. 2.5 mg of lysate from light cells and 2.5 mg of lysate from heavy cells was mixed and

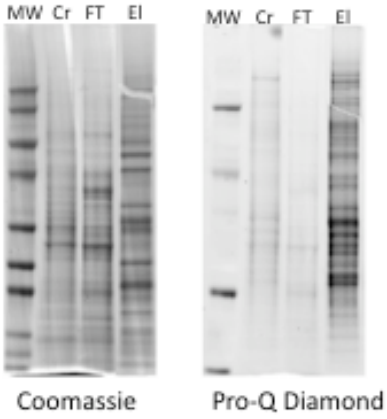

Figure 2. Phosphoprotein enrichment of proteins from MCF-7/HER2-18 cells. MCF-7/HER2-18 cells were split into two equal samples and grown in either heavy or light SILAC media. The heavy cells were then treated with 10 nM Tamoxifen and the light cells with ethanol as control, for a total of 30 minutes. The samples were lysed and mixed at 1:1. Phosphoproteins were isolated using a phosphoaffinity column (Pro-Q Diamond, Invitrogen/Molecular Probes). Lysate (L), flowthough (FL) and Eluate (E) from the phosphoaffinity column were subjected to SDS-PAGE and the gel stained with Imperial Coomassie to visualize proteins and Pro-Q Diamond fluorescent stain to visualize phosphoproteins. Representative figure for MCF-7/HER2-18 and MCF-7 cells.

the combined lysate was loaded onto pre-equilibrated Pro-Q Diamond resin, the column washed and phosphoproteins eluted. The lysate, flow-through and eluate were concentrated in 10 kDa MWCO Vivaspin concentrators at 4 °C and washed with 50 mM Tris, pH 7.5. The samples were mixed with Laemmli buffer and incubated at 95°C for 5

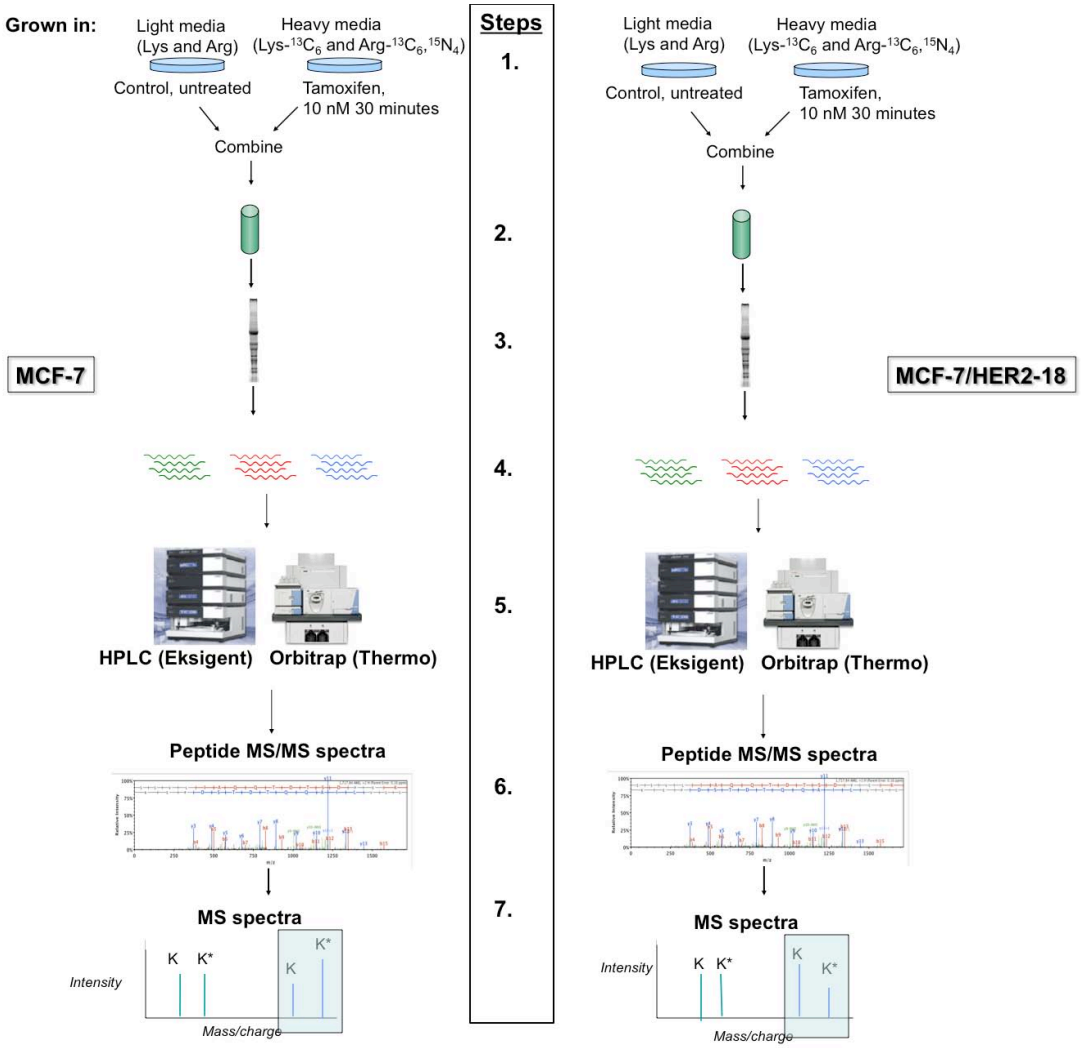

Figure 1. Scheme for differential phosphoprotein profiling. Two cell lines were used for analysis, MCF-7 and MCF-7/HER2-18. (1) One sample is grown in media with stable isotope labeled arginine (Arg) and lysine (Lys) (heavy sample) and another grown in regular media (light sample). Heavy sample is treated with 10 nM Tamoxifen for 30 minutes, the light sample is untreated control. Samples are then combined, subjected to (2) phosphoenrichment (Pro-Q Diamond resin, Invitrogen/Molecular Probes), separation by (3) SDS-PAGE (cut into 18 sections). The samples are then (4) digested and peptides extracted and subjected to (5) reversed phase nanoLC-MS/MS. Peptide and protein identification from (6) MS/MS spectra using Mascot, X!Tandem and compiled in Scaffold. Relative abundance calculated from MS spectra (7) using XPRESS in CPAS. Experiments were repeated identically except tamoxifen treatment was performed on the light sample (gel B in table 1). Peptides whose abundance ratios differ between MCF-7 and MCF-7/HER2-18, represented by blue peptide in shadowed box, are the ones of interest.

min before loading on NuPAGE 2-12% gradient gels. The gel was stained for

phosphoproteins using Pro-Q Diamond stain and subsequently for proteins with Imperial Coomassie stain. Coomassie stained protein was visible in all three fractions including the flow through (Figure 2, see previous page). The dark staining in the eluate fraction and the scarcity of phosphoproteins in the flowthrough fraction shows that the Pro-Q Diamond resin selectively binds phosphoproteins.

MASS SPECTROMETRY OF THE ENRICHED PHOSPHOPROTEINS

Proteins were extracted for mass spectrometry analysis from the ProQ elution gel lane of the SDS-PAGE gel (Figure 2, elution lane). Briefly, the molecular weight region above 10 kD was divided into 20 sections, about 0.5 cm each. The top two and second two sections were combined, giving a total of 18 sections. Each section was cut into small pieces, each ~1 mm^3. Sections were washed in water and completely destained using 100 mM ammonium bicarbonate in 50% acetonitrile. A reduction step was performed by addition of 100 µl of 50 mM ammonium bicarbonate pH 8.9 and 10ul of 10 µM TCEP and allowed to reduce in 37 ºC for 30 min. The proteins were alkylated by adding 100 µl of 50 mM iodoacetamide and allowed to react in the dark for 40 min. Gel sections were washed in water, initially dried with acetonitrile followed by a SpeedVac step of 30 min. Digestion was carried out using sequencing grade modified trypsin (40 ng/ml, Promega) in 50 mM ammonium bicarbonate. Sufficient trypsin solution was added to swell the gel pieces, which were kept in 4º C for 45 min and then incubated at 37º C overnight. Sections containing proteins larger than 150 kD were pre-digested with Lys-C (0.25 mg/ml, Princeton Separations) in 6-8 M Urea overnight at 25 ºC, diluted to final concentration of less than 2 M Urea then digested with trypsin as described above. Peptides were extracted from the gel pieces with 5% formic acid.

All mass spectrometry was performed in the Mayo Proteomics Research Center, on Thermo LTQ-Orbitrap Hybrid FT Mass Spectrometers. The peptide samples were loaded to a 0.25 µl C8 trapping cartridge OptiPak custom-packed with Michrom BioResources Magic C8, 5 µm, 200A, washed, then switched in-line with a 20 cm by 75 um C18 'packed spray tip' nano column packed with Magic C18AQ, 5 µm, 200A, for a 2-step gradient, where mobile phase A is water/acetonitrile/formic acid 98/2/0.2 and mobile phase B is acetonitrile/isopropanol/water/formic acid 80/10/10/0.2. Using a flow rate of 350 nl/min, a 90 min, 2-step LC gradient was run from 5% B to 50% B in 60 min, followed by 50%-95% B over the next 10 min, hold 10 min at 95% B, back to starting conditions and re-equilibrated. The samples were analyzed via electrospray tandem mass spectrometry (LC-MS/MS) on the LTQ-Orbitrap using a 60,000 RP Orbi survey scan, m/z 375-1950, with lock masses, followed by 5 LTQ CAD scans with isolation width of 1.6 Da on doubly and triply charged-only precursors between 375 Da and 1500 Da. Ions selected for MS/MS were placed on an exclusion list for 60 s using low mass exclusion of 1.0 Da, high mass exclusion of 1.6 Da.

The mass spectrometry data were converted to .mgf files via .mzXML intermediates and searched using Mascot using the SILAC (MD) quantitation parameter. A fragment ion mass tolerance of 50 ppm and a parent ion tolerance of 0.6 Da were specified. Oxidation of methionine, phosphorylation (S, T, Y) and carbamidomethyl (C) were specified as variable modifications. Mascot results were loaded into Scaffold (Proteome Software), which uses Peptide and Protein prophet to

calculate probabilities. Scaffold also conducted an X!Tandem search using the parameters used for Mascot.

Comparative Proteomics Analysis System(CPAS) is a open-source analytic system based on the modules developed in the Trans Proteomic Pipeline from Institute of Systems Biology (Seattle) [30]. CPAS was used to perform quantitation on the data from mzXML files. The analysis pipeline involved performing X!Tandem searches (using the parameters described above), converting the results to .pepXML format, processing by Peptide Prophet for statistical evaluation of peptide identifications and Xpress software for relative peptide quantification. The peptide results from all 18 sections were exported and combined into one excel file. Proteins were compiled and protein averages calculated using a Perl script provided by the Hanash lab at Fred Hutch (Seattle). Experiments were performed in duplicate, gel A where heavy cells treated with tamoxifen and light were untreated and gel B where light cells were treated with tamoxifen and heavy were untreated (Table 1).

Table 1. Overview of mass spectrometry experiments.

Cells	**Name**	**Tamoxifen treatment**	**Control**	**# Sections**	**Status**
MCF-7	GelA	Light	Heavy	18	Completed
MCF-7	GelB	Heavy	Light	18	Completed
MCF-7 /HER2-18	GelA	Light	Heavy	18	Completed
MCF-7 /HER2-18	GelB	Light	Heavy	18	Awaiting mass spectrometry analysis

RESULTS PHOSPHOPROTEIN PROFILING OF CONTROL AND TAMOXIFEN TREATED MCF-7 AND MCF-7/HER2-18 CELLS

Proteomic analysis of tamoxifen response in MCF-7 cells resulted in identification of over 1500 proteins (protein probability >99%, peptide probability >95%, requiring a minimum of 2 unique peptides per protein identification). Quantitation was performed and only protein ratios with less than 10% standard deviation between gelA and gelB (Table 1) were averaged and included in further analysis. The vast majority of proteins did not change substantially in abundance (Figure 3). About 20 proteins were identified that decreased >25% and about 30 proteins that increased >25% in the tamoxifen treated sample. Gene ontology analysis of these proteins reveals that they are involved in several important processes such as protein transport, DNA repair, signal transduction and protein biosynthesis.

Phosphoprotein profiling on MCF-7/HER2-18 tamoxifen resistant cells resulted in identification of over 1400 proteins (protein probability >99%, peptide probability >95%, requiring a minimum of 2 unique peptides per protein identification). Among the proteins identified were HER2 kinase, as expected since it is over-expressed in the cell line. Quantitation revealed that the vast majority of proteins did not change substantially in abundance. 5 proteins were identified that decreased >25% in the tamoxifen treated sample and 8 proteins that increased >25% in the tamoxifen treated sample. Gene

ontology analysis of these proteins reveals that the proteins are involved in several important processes such as DNA repair, protein transport and signal transduction.

I have compared the results from MCF-7 to MCF-7/HER2-18 phosphoprotein profiling of tamoxifen response and identified 26 proteins that respond to tamoxifen differently. All but three of these proteins are known to be phosphorylated and at least one of the three proteins is known to bind to a phosphoprotein and could thus have been purified on the Pro-Q Diamond resin as a phosphoprotein complex. I have divided the proteins into 3 categories:

A. Opposite responses to tamoxifen in the two cell lines

The most obvious proteins of interest are those that respond differently to tamoxifen treatment in the sensitive and resistant cell lines. 6 proteins are identified in both cell

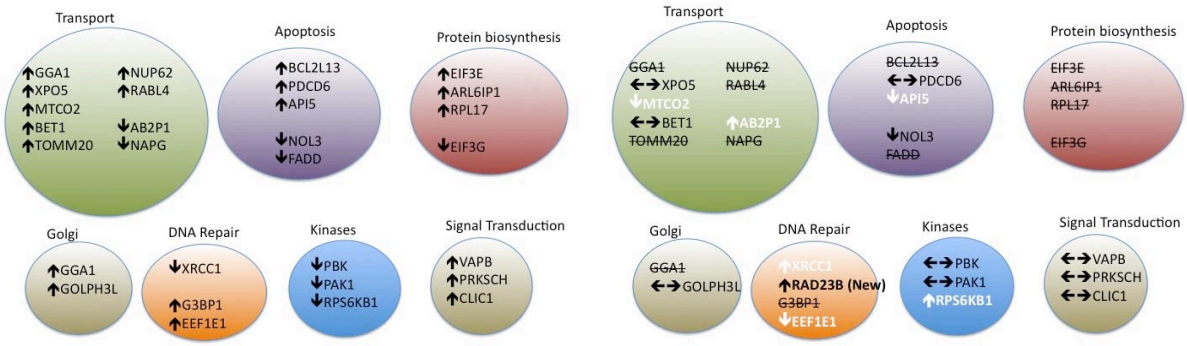

Figure 4. A (left). Proteins that change in response to tamoxifen in MCF-7 cells. B(right). Proteins that change in response to tamoxifen in MCF-7/HER2-18 cells (tamoxifen resistant). Proteins in white are those whose response differs between the two cell lines. Arrows reflect changes between control and tamoxifen sample, ie. up indicates that there was more protein in the tamoxifen sample, left and right arrows indicate that the protein did not change in abundance. Proteins with a single line over the name indicate that they were identified in the MCF-7 sample but not in the MCF-7/HER2-18 sample.

lines and have an opposite response to tamoxifen (for example up in one, down in the other cell line). These proteins are colored white in Figure 4. AB2P1 and MTCO2 are proteins involved in transport, API5 is a protein involved in apoptosis, XRCC1 and EEF1E1 are DNA repair proteins and RPS6KB1 is a protein kinase (Table 2). I have selected two proteins to follow up on. First, API5 is an anti-apoptotic protein by virtue of suppressing cleavage of DNA and is also known to suppress E2F stimulated apoptosis. Upregulation of API5 gene expression levels was found to correlate with breast cancer patients that do not respond to tamoxifen treatment (de-novo resistance) [31]. We found that the phosphorylation and/or protein levels of API5 were increased in MCF-7 cells and decreased in MCF-7/HER2-18 cells in response to tamoxifen treatment. API5 has been reported to be serine phosphorylated but no phosphospecific antibodies are available. How the function and/or activity of API5 is affected by phosphorylation is unclear.

Second, RPS6KB1, the mammalian target of rapamycin (mTOR) effector P70 S6 protein kinase (S6K), is amplified in approximately 10% of breast cancers. RPS6KB1 gene amplification correlates with HER2 overexpression in breast tumors, possibly due to co-amplification of RPS6KB1 with HER2 (both genes reside on 17q) [32]. Since our tamoxifen resistant cell line does overexpress HER2, an increase in RPS6KB1 protein amounts is to be expected. The decrease in RPS6KB1 in MCF-7 cells will be investigated. I have obtained antibodies to API5 and RPS6KB1. I am currently optimizing Western blot conditions for API5 antibody as the initial experiments did not result in a API5 protein signal.

B. Response to tamoxifen in one cell type but not the other

Another interesting type of response is change in protein due to tamoxifen in one cell line but no response in the other. 9 proteins, PAK1, PDCD6, XPO5, BET1, PBK, VAPB, PRKSCH, CLIC1, GGA1, changed in response to tamoxifen in the MCF-7 cells but stayed unchanged in response to tamoxifen in the MCF-7/HER2-18 cells. Interestingly, PAK1 kinase is known to directly phosphorylate ER and amplification of PAK1, especially nuclear PAK1, in ER+ breast cancer patients has been found to correlate with lower survival rates [33] [34]. In addition, the drug FK228 reduces PAK1 activity without changing protein levels and resulted in growth inhibition in both tamoxifen sensitive and resistant cell lines [35]. Thus this phosphoprotein profiling methodology has identified a protein known to be important for breast cancer patient survival. I have obtained a PAK1 pan-antibody and several PAK1 phosphospecific antibodies to identify if PAK1 protein levels decreased in MCF-7 cells or if the phosphorylation of the protein decreased. Initial Western blots did not produce a signal, I am currently optimizing Western blot conditions.

C. Response to tamoxifen in one cell line, but not detected in the other cell line

12 proteins, BCL2L13, FADD, RABL4, NUP62, EIF3E, ARL61P1, RPL17, EIF3G, G3BP1, GGA1, TOMM20, NAPG, changed in MCF-7 cells but were not detected in the MCF-7/HER2-18 cells. Although this category of proteins could potentially be very interesting i.e. protein phosphorylated in one cell line but not in the other, it could also lead to false positives. Indeed, the lack of detection in any mass spectrometry experiment does not necessarily mean that the protein is not there. Thus the protein might behave identically in both cell lines but the protein was not detected by mass spectrometry in one cell line, possibly due to increased expression of other proteins that were selected for mass spectrometry instead. One promising candidate with this type of response is FADD, an apoptotic adaptor molecule that recruits activated Caspase 8 or 10 to activated Fas and TNFR-1 (Tumor Necrosis Factor) receptors.

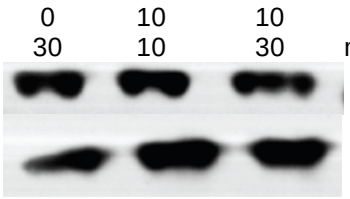

Figure 5. FADD protein is detected in both MCF-7 and MCF-7/HER2-18 cells. 60 µg of protein lysate from MCF-7 or MCF-7/HER2 cells treated with tamoxifen or left untreated was loaded onto a 4-12% Nu-PAGE gradient gel (Invitrogen). The Western blot was performed using 1:1000 anti-FADD antibody in 5% milk in TBST at 4 degrees overnight and 1:5000 anti rabbit in 5% milk in TBST for 1 hour at room temperature.

Phosphorylation of FADD on Serine194 is statistically different between breast tumor epithelial cells and matched undissected breast tissue [36]. Expression of a dominant negative mutant form of MKK7, a kinase upstream of JNK, or mutant FADD (S194A) in MCF-7 cells suppressed the cytotoxicity of long-term tamoxifen treatment [37]. I performed a Western blot with FADD antibody showing that FADD protein was detected in both MCF-7 cells and MCF-7/HER2-18 cells (Figure 5). Thus, although FADD was not detected in the MCF-7/HER2-18 it is not due to the protein being absent. A second possibility is that FADD is only phosphorylated in MCF-7 cells but not in MCF-7/HER2-18 cells. I have also obtained anti-FADD phosphoSer194 antibody and am optimizing conditions for Western analysis.

I will continue to perform manual validation on each of these proteins to confirm the protein ratio, compare the protein hits to tamoxifen responsive proteins identified in the literature and finally confirm the results by biological validation (Western Blots, siRNA and overexpression studies). Although much has been accomplished, there is also more work ahead to validate these possible predictors of tamoxifen resistance.

Computational solutions to complex signaling analysis

Once samples have been labeled with stable isotope, each peptide appears as a doublet. This easily distinguishes peptides from background. In addition, since the isotope is added to the C-terminal (in SILAC and $^{18}0$ labeling) C-terminal fragment ions (y-ions) are shifted (light and heavy forms) from non-labeled and non-shifted N-terminal fragment ions (b-ions). Utilizing this information Sam Volchenboum and I have developed a fast and reliable method for automated validation of Mascot search results from high accuracy mass spectrometry data. We can identify isotopic pairs within searched Mascot data (dat file), and these pairs represent the highest confidence peptide matches. Our software, termed Validator, demonstrated a false discovery rate of only 2% while retaining most high-Mascot scoring peptides and eliminating most low-scoring ones. Finally, we demonstrated that our software identifies peptide pairs based only on their difference in precursor mass owing to the presence of the stable isotope label using no Mascot-specific information. We were able to corroborate 81% of identified peptide pairs using conventional database search engines and published the paper in Journal of Molecular and Cellular Proteomics [38] (the paper in its entirety is found in the appendix). We are currently working on a second publication; describing a program we have termed Identifier. Identifier takes the proteome of an organism, for example yeast, and generates in silico digested peptides listing the peptide sequence, mass and the identity and mass of b- and y-ions. Thus the workflow will involve using Validator to identify peptide pairs from the raw data and comparing the mass and fragmentation patterns of peptides to the in silico digested proteome.

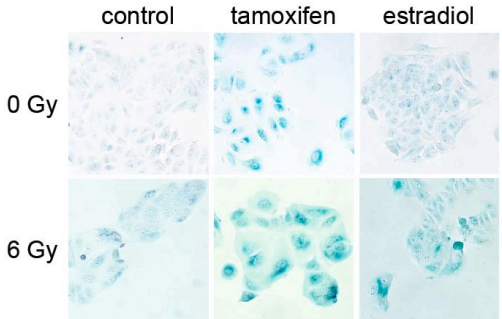

Figure 6. IR + TAM induces MCF-7 cell senescence. Note large, flat cells and increased SA-ß-gal staining in TAM compared to control or

This allows for very rapid analysis of mass spectrometry data and represents a novel method of protein identification that can be used instead of or in addition to conventional database search engine methods.

Irradiation and tamoxifen induced MCF-7 cell senescence

Tumor cell senescence, like apoptosis, has been proposed as a desirable outcome of radiation therapy and chemotherapy [26, 27] but agents that promote senescence have yet to be described. Estrogen was recently shown to decrease IR induced senescence [28]. Thus, we decided to test if tamoxifen could inhibit IR induced senescence and potentially be used as a radiosensitizer. In addition to the phosphoprotein profiling described above, I examined whether tamoxifen could sensitize cells to radiation. In collaboration with Dr. Elena Effimova and Dr. Ralph Weichselbaum, we observed IR-induced senescence in MCF-7 cells in vitro and demonstrated that tamoxifen strongly promotes DNA damage-induced senescence in MCF-7 cells (Figure 6). Previously, Drs. Wechselbaum and Effimova had demonstrated that the PARP inhibitor ABT-888 promotes senescence by blocking DSB repair (data not shown, manuscript in preparation). We then tested tamoxifen and ABT-888 together and found a synergistic response (not shown). Briefly, MCF-7 cells were plated on 60 mm plates, treated with DMEM with 5% stripped media for 4 days followed by 48 hours of doxycyclin treatment to induce expression of GFP-53BP1. Cells were irradiated with 6 Gy and treated with ethanol, 10 nM tamoxifen, 10 nM estrogen and/or 10μM ABT-888 in DMEM media (previously described) for a period of 6 days. Senescence was detected by examining morphology (large cells) and by beta-galactosidase (SA-β-gal) staining (Senescence beta-Galactosidase staining kit from Cell Signaling). We observed a significant enhancement of cell senescence in MCF-7 breast cancer cells treated with IR + tamoxifen over IR alone. We hypothesize that tamoxifen blocks ERα repression of DNA damage-dependent cell signaling and restores activity of the p53 pathway to allow persistent DNA damage to promote terminal cell cycle arrest. We are currently repeating the experiments and will perform Western blots to examine p53, p21 and y-H2AX status in MCF-7 cells treated with a combination of IR, TAM and/or ABT-888 compared to untreated cells.

Key Research Accomplishments

- I have performed phosphoprotein enrichment from tamoxifen treated and control untreated samples from tamoxifen sensitive (MCF-7) and tamoxifen resistant (MCF-7/HER2-18) cell lines. The experiment was performed twice for each cell line.
- Each experiment identified over 1400 proteins, over 30% of which are known phosphoproteins.
- I have compared the results from MCF-7 to MCF-7/HER2-18 phosphoprotein profiling of tamoxifen response and have divided the results into 3 categories:
    - A. Opposite responses to tamoxifen in the two cell lines

      6 proteins, AB2P1, MTCO2, XRCC1, EEF1E1, RPS6KB1, API5 were identified in both cell lines and have an opposite response to tamoxifen (for example up in one, down in the other cell line).
    - B. Response to tamoxifen in one cell type but no change in the other

      9 proteins, PAK1, GGA1, PDCD6, XPO5, BET1, PBK, VAPB, PRKSCH, CLIC1, changed in response to tamoxifen in the MCF-7 cells but stayed unchanged in response to tamoxifen in the MCF-7/HER2-18 cells.
    - C. Response to tamoxifen in one cell line, but not detected in the other cell line

      12 proteins, BCL2L13, FADD, RABL4, NUP62, EIF3E, ARL61P1, RPL17, EIF3G, G3BP1, GGA1, TOMM20, NAPG, changed in MCF-7 cells but were not detected in the MCF-7/HER2-18 cells.
- In collaboration with Sam Volchenboum, Instructor in Pediatrics and the Computational Institute at the University of Chicago, I developed a fast and reliable method for automated validation of Mascot search results from high accuracy mass spectrometry data which was published the paper in Journal of Molecular and Cellular Proteomics.
- In collaboration with Prof. Wechselbaum's lab in the department of Radiation Oncology at the University of Chicago I observed a striking synergistic interaction between ionizing radiation (IR) and tamoxifen in a model for combining high dose radiation therapy (RT) with hormone therapy for breast cancer

Reportable Outcomes

A. Talks and poster presentations

1. **Cancer Biology Training Consortium, Chairs and Program Directors Retreat and Annual Meeting (CABTRAC)** in Basin Harbor Resort, Vermont, September 30th-October 2 2007. Presented poster entitled: "Phosphoprotein profiling for quantitative analysis of phosphorylated proteins"
2. **American Association for Cancer Research (AACR) Annual Meeting.** San Diego, California, April 10-15th, 2008. Presented poster entitled: "Differential phosphoprotein profiling of Tamoxifen response".
3. **Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting** in Baltimore, MD in June 25-28th, 2008. Presented poster entitled: "Differential phosphoprotein profiling of Tamoxifen response".
4. **University of Chicago Annual Molecular Biosciences Retreat**, Galena, IL November 7-9, 2008. Oral presentation titled: "Differential Phosphoprotein Proteome Profiling of Tamoxifen Response"
5. **29th Annual Minisymposium on Reproductive Biology.** Evanston, IL, October 6th, 2008. **Presented poster entitled:** Presented poster entitled: "Differential phosphoprotein profiling of Tamoxifen response".
6. **Midwest Breast Cancer Research Symposium.** Iowa City, Iowa. July 17-19th, 2009. Presented poster entitled: "Differential phosphoprotein profiling of Tamoxifen response".
7. **Gordon Conference: Hormone Action In Development & Cancer.** Holderness, NH, July 26-31st, 2009. Presented poster entitled: "Differential phosphoprotein profiling of Tamoxifen response".

B. Grant application based on research funded by this grant

Kristjansdottir, K., K99/R00 Pathway to Independence Grant Resubmission July 12th 2009. "Systems analysis of breast cancer signaling pathways".

C. Publications and manuscripts in preparation

Volchenboum, S.L., **Kristjansdottir,** K., Wolfgeher, D., and Kron, S.J. Rapid validation of Mascot search results via stable isotope labeling, pair picking and deconvolution of fragmentation patterns. *Mol Cell Proteomics.* 2009. **8**, pp. 2011-22.

Kristjansdottir, K., and Kron, S.J. Stable isotope labeling for protein quantitation by mass spectrometry. Review. *In preparation.*

Volchenboum, S.L., **Kristjansdottir, K.** and Kron, S.J. Rapid identification of stable isotope labeled peptides via stable isotope labeling and comparison of experimental and calculated y-ions. *In preparation.*

Kristjansdottir, K., Greene, GL. And Kron. S.J. Phosphoprotein profiling of tamoxifen response in MCF-7 cells. *In preparation.*

Effimova, E., **Kristjansdottir, K.**, Wechselbaum, R. and Kron, SJ. Tamoxifen synergized with iradiation and ABT-888 to promote cell senescence in MCF-7 cells. *In preparation.*

Conclusion

I have developed a method for comparison of global phosphoprotein profiles. The methodology involves stable isotope labeling, a phosphoprotein affinity step, 1-D SDS-PAGE and LC-MS/MS. I have performed phosphoprotein profiling of MCF-7 (tamoxifen sensitive) and MCF-7/HER2-18 (tamoxifen resistant) cells as a result of tamoxifen treatment. Comparing the results identified 26 proteins that respond to tamoxifen differently in MCF-7 (tamoxifen sensitive) and MCF-7/HER2-18 (tamoxifen resistant) cells. All but three of these proteins are known to be phosphorylated. Several proteins have previously been described as being involved in generation of tamoxifen resistance including FADD and PAK1, showing that phosphoprotein profiling is capable of identifying proteins relevant to tamoxifen resistance. I have selected several proteins for ratio validation using Western blots and pan-antibodies (API5, RPS6KB1) and phosphospecific antibodies when possible (PAK1, FADD). Future directions include validating the potential predictors of tamoxifen response by Western blots, shRNA and/or overexpression of predictors in MCF-7 cells and analysis of tamoxifen sensitivity. Findings will be confirmed in other cell line pairs (sensitive and resistant) to establish a common mechanism of tamoxifen resistance.

In collaboration with Sam Volchenboum, Instructor in Pediatrics and the Computational Institute at the University of Chicago, I developed a fast and reliable method for automated validation of Mascot search results from high accuracy mass spectrometry data which was published the paper in Journal of Molecular and Cellular Proteomics. We are currently working on expanding the software to identify peptide sequences and provide a novel methodology for protein identification from mass spectrometry data.

We observed a striking synergistic interaction between ionizing radiation (IR) and tamoxifen in a model for combining high dose radiation therapy (RT) with hormone therapy for breast cancer. We hypothesize that tamoxifen blocks ERα repression of DNA damage-dependent cell signaling and restores activity of the p53 pathway to allow persistent DNA damage to promote terminal cell cycle arrest. We are currently performing Western blots to examine p53, p21 and y-H2AX status in MCF-7 cells treated with a combination of IR, TAM and/or ABT-888 compared to untreated cells.

KRISTJANSDOTTIR, KOLBRUN

Statement of Work

Phosphoprotein profiling of SERM response in breast cancer cell lines

Task 1. To finish data analysis on phosphoproteomics on tamoxifen response in MCF-7 and MCF-7/HER2-18 cells (Months 1-4).

a. Analyze duplicate MCF-7/HER2-18 proteomic experiment. (Month 1-2)
b. Perform manual validation of proteomic results identifying markers of tamoxifen response (2 cell lines, 2 duplicates each) (Month 3).
c. Compare global protein lists from each experiment, generate statistics on reproducibility. (Month 4)
d. Finish analyzing available proteomic, gene expression and protein abundance databases and literature and compare our results with previous findings. (Month 4)

Task 2. Biological validation of hits from Task 1 (Months 5-7):

a. Finish Western blots examining the levels of FADD and p-FADD. Perform time course from cell lines and immunoprecipitations to see if I can get the p-FADD antibody to work. (Month 5).
b. Test abundance of selected other hits using WB and/or immunoprecipitations. (Month 6).
c. Perform RT-PCR on selected hits from Task 1 (FADD, API5, XRCC1, BET1, DTYMK, PBK, PAK1 and more). (Month 7).

Task 3. Further proteomic analysis. (Months 8-12).

a. Am awaiting proteomic analysis from MCF-7/HER2-18 cells treated with 1 μM tamoxifen for short period (high concentration, low time) and from 10 nM tamoxifen for overnight period. Analyze data using CPAS, Mascot and Scaffold as with previous data. (Months 8-10)
b. Compare these results to the results from Task 1. Both proteins and pathways will be compared to see if longer time allows for signal transduction, that we can follow. (Month 11-12)

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Appendices

Manuscript: Volchenboum, S.L., **Kristjansdottir,** K., Wolfgeher, D., and Kron, S.J. Rapid validation of Mascot search results via stable isotope labeling, pair picking and deconvolution of fragmentation patterns. *Mol Cell Proteomics.* 2009. **8**, pp. 2011-22.